

1992

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Henderson, Eric, "Imaging and nanodissection of individual supercoiled plasmids by atomic force microscopy" (1992). *Zoology and Genetics Publications*. 35.

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Imaging and nanodissection of individual supercoiled plasmids by atomic force microscopy

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Received November 12, 1991; Revised and Accepted December 17, 1991

ABSTRACT

The atomic force microscope (AFM) was used to image supercoiled plasmid DNA deposited on a mica surface in either a hydrated or desiccated state. Hydrated plasmid was precisely cut by the scanning tip at a location determined by the instrument operator. Small pieces of DNA (100–150 nm in length) were excised and deposited adjacent to the dissected plasmid, demonstrating that it is possible to remove and manipulate genomic DNA fragments, unresolvable by light microscopy, from defined chromosomal locations by AFM.

INTRODUCTION

The atomic force microscope (AFM) is capable of imaging surfaces at extremely high, sometimes atomic, resolution [1]. In the AFM a sharp tip on the end of a reflective cantilever is scanned in the X and Y directions over a surface. As the tip encounters surface features deflections in the Z axis are measured by an optical lever system [2]. When small forces are applied to the sample the AFM is able to provide 3-dimensional images of the substrate being scanned. When larger forces are used, however, the tip can cause mechanical deformation and disintegration of the sample. Controlled application of force applied by the scanning tip can allow dissection of the sample at a resolution heretofore unobtainable. This is vividly illustrated in a recent report describing the dissection of a single gap junction by in the AFM to reveal molecular structures within the junction [3].

Microdissection of chromatin is a useful tool for cloning specific regions of a particular chromosome. This is accomplished by skilled use of fine glass needles under the control of micromanipulation devices [4]. Typical sizes of the pieces dissected this way from polytene chromosomes can be as small as 0.5 μ . However, this size is limited by the optical resolution of a light microscope and the size of the glass needle. Two recent reports suggest that the AFM can be used in a similar fashion on individual DNA molecules with the dissection greatly improved in terms of both speed and precision [6, 7]. To accomplish this, DNA must be readily imaged in the AFM and be susceptible to scission by the scanning tip. This report shows that it is possible to image supercoiled plasmid DNA in both a hydrated and desiccated state by AFM and that small regions of the plasmid can be easily excised by the scanning tip, thus further demonstrating the feasibility of using the AFM for physical manipulation of genetic material.

MATERIALS AND METHODS

Plasmid Preparation and Deposition

The two plasmids used in these experiments were typical bacterial episomes 9.1 and 9.7 kb in length. Supercoiled plasmids were isolated by isopycnic centrifugation in CsCl gradients containing ethidium bromide [5]. Supercoiled plasmids dissolved in TMN (10 mM Tris base, pH 7.2, 5 mM MgCl₂, 50 mM NaCl, 1 mM EDTA) or in water at a concentration of 1–5 μ g/ml were deposited on freshly cleaved mica (Pelco International, Tustin CA) in a 10 to 20 μ l droplet and allowed to stand for 5 minutes. The drop spread to cover the mica (about 0.3 \times 0.4 cm) immediately. After five minutes residual liquid was blotted from the edge taking great care to avoid contact with the surface to be imaged. The mica was then lightly dragged through a solution of 10 mM NH₄OAc 3–4 times to remove nonvolatile salt. At this point one of two procedures was followed. The sample was either air dried for 5–10 minutes and immediately imaged in the AFM or it was placed in a vacuum oven at 80°C for 30–60 minutes and then imaged immediately upon removal.

AFM Imaging and Plasmid Dissection

Images were collected using a Nanoscope II AFM equipped with A and D scanners (Digital Instruments, Inc., Santa Barbara, CA). Scanning tips used were 'Nanoprobes' (Digital Instruments, Inc.) constructed of Si₃N₄. The cantilevers on which the tips were grown were usually 200 μ in length ($k = 0.12$ N/m) although occasionally a 100 μ cantilever ($k = 0.58$ N/m) was used. Both height (constant force) and force (cantilever deflection signal) mode were used. For highest resolution, gains were adjusted to reveal the most surface detail in the force mode (this is termed error signal mode). For dissection of plasmid, gains were minimized to reduce compensatory movement of the piezo crystal when the tip encountered the plasmid. At the desired position the scan was limited to the X direction only and the setpoint increased to 10 volts (thus applying increased force to the sample). Several passes were permitted before reactivating the Y scan direction and collection of the image. When possible (i.e., when a good force curve was obtainable), imaging forces were calculated according to the procedure outlined in the Nanoscope manual and were typically in the 1–10 nN range. In general, for imaging the force was reduced by lowering the setpoint until detachment from the surface was observed and then operating about 1 volt above that level (typically 0.0 to –2.0 volts). The relative humidity, recently shown to be a critical factor in stabilization of DNA on mica [7] was typically 50% although this parameter was not controlled in this study and was therefore variable.

RESULTS

Imaging Supercoiled Plasmids by AFM

Supercoiled plasmids were deposited on freshly cleaved mica by the two methods described in the MATERIALS AND METHODS section. Images similar to those shown in Figure 1 were reproducibly obtained. If plasmid DNA was deposited and dried under ambient conditions (room temperature, relative humidity approximately 50%) relatively poorly resolved images were observed (Figure 1A). The molecules were sufficiently stable to resist being swept out of the field of view but were clearly susceptible to deformation by the scanning tip. Presumably the coat of hydration on the mica and the DNA prevents firm attachment thereby permitting the DNA to roll during imaging. Furthermore, hydrated molecules are likely to have a softer surface that is more deformable. The effects of hydration upon image acquisition by AFM have been discussed by others [6,7]. In contrast to hydrated molecules, samples that had been dried and baked onto the surface were extremely stable (Figure 1B). This treatment gives much better indications of surface features

and topology of the plasmids. Frequently these molecules had a branched appearance similar to that seen in electron microscopic images of supercoiled plasmids.

Observation of relaxed circular species was very rare in this study although two recent reports demonstrate frequent observation of relaxed circular plasmid by AFM [6, 7]. This discrepancy is likely to be due to differences in the method of preparation of plasmid DNA and deposition conditions. An example of a relaxed circular plasmid is shown Figure 1C. Like the supercoiled plasmids, the width of the DNA is exaggerated (see the DISCUSSION section), however the contour length is within 6% of that expected (3298 nm expected for a 9.7 kb plasmid, 3490 nm measured).

Dissection of Single Supercoiled Plasmid Molecules

By increasing the imaging force it was possible to surgically remove small portions of hydrated supercoiled plasmids with the AFM tip (Figure 2). Figure 2A, panel 1, shows the DNA prior to application of increased imaging force. Figure 2A, panel 2,

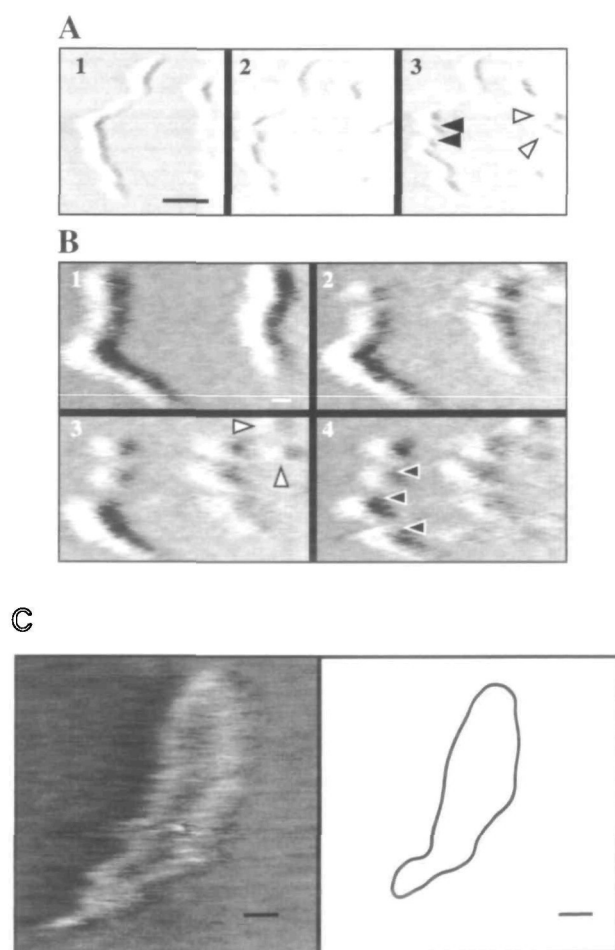


Figure 1. Supercoiled plasmid DNA imaged by AFM. In all images presented the lighter features are elevated relative to darker features. A. Supercoiled plasmid deposited on mica and dried at room temperature and humidity. The blurry image is a consequence of sample movement, presumably due to interaction with the scanning tip. Scale bars = 1 μ . B. Supercoiled plasmid dried onto mica at 80°C and under vacuum. Note that the images reveal branching expected for supercoiled plasmids. Scale bars = 1 μ . C. AFM image and tracing of a relaxed circular plasmid deposited on mica and dried at room temperature and humidity. Scale bar = 200 nm.

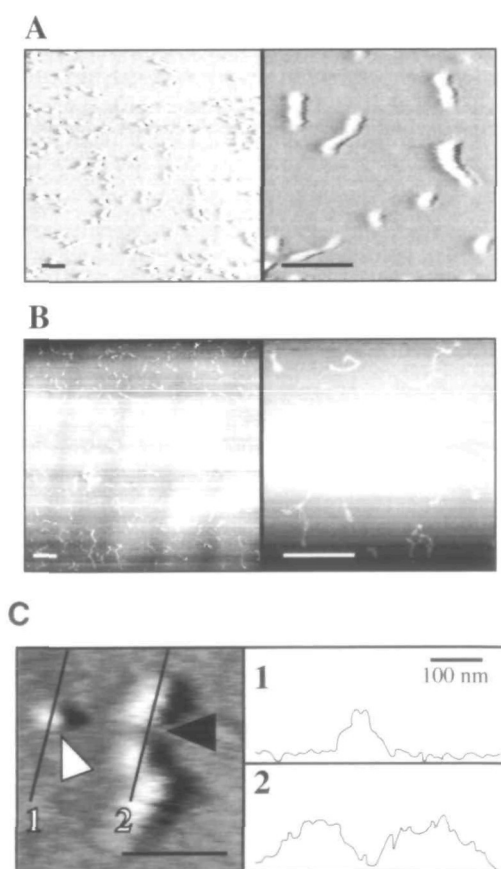


Figure 2. Dissection of supercoiled plasmid by AFM. A and B show two examples of sequential dissections and image acquisition scans. Panel 1 shows the plasmids prior to dissection, subsequent panels show the results of sequential dissection attempts. Black arrows indicate the gaps left in the plasmid after scanning at a fixed Y position under relatively high force. The white arrows indicate pieces of DNA removed from the plasmids. For detailed description see text. Scale bars for A and B = 500 nm and 100 nm respectively. C. Image of a single dissection (left hand panel) with tracings of sections (right hand panel) through the plasmid (black arrow) and the excised piece of DNA (white arrow). The angle of the sections is indicated by the black lines and the lines are numbered to correspond to the sections. Scale bar in the image = 500 nm. Note that the scale of the sections is different from that of the image.

shows the same molecule after disabling scanning in the Y direction at a desired location and increasing the force for several scans in X (for details see the MATERIALS AND METHODS section). A gap is apparent in the molecule at the position of Y disable and the piece removed from this gap is near the right edge of the field. Figure 2A, panel 3, shows the results of repeating this process at a different location. Two gaps (black arrows) and two excised pieces (white arrows with black boarder) are apparent. Figure 2B, panels 1–4, shows a second example in which two molecules are visible. In this case it is not clear from which plasmid the indicated excised fragments originated. Applying this same method to desiccated plasmids gave less favorable results. Gaps could be produced in the plasmids but the excised fragments were not visible (not shown).

The size of the DNA fragments excised (and the resulting gap in the plasmids from which they originated) was in the range of 100–150 nm (Figure 2C). This size is smaller than that resolvable by light microscopy and corresponds to 300–400 bp of linear duplex DNA, less than the length of a typical gene. Since the plasmids were supercoiled, and thus folded back upon themselves, the fragments dissected were more likely in the range of 1000 bp long, still a gene-sized piece.

DISCUSSION

Images of supercoiled plasmids under conditions of relatively high and low hydration were reliably obtained by AFM. In this and other studies the molecular dimensions, particularly width in the case of a linear polymer like DNA, were exaggerated [6, 7, E. Henderson, unpublished data]. This is, in part, because the image observed is a convolution of the tip and the sample surfaces. Since conventional AFM tips are not infinitely sharp (i.e., they have a radius of curvature) molecules appear wider than they actually are. This phenomenon is described in detail by Bustamante et al. [6] and Vesenka et al [7]. In addition, sample movement during image acquisition adds to this effect. In the study described here hydrated molecules appeared thicker and less branched than desiccated molecules. Since the mechanical properties of the AFM were identical during imaging of hydrated and desiccated plasmids, the physical differences observed were due to differences in the sample exclusively. Sample movement during imaging undoubtedly contributed to the blurred images observed with hydrated plasmids. It is also possible that conformational changes that result from changes in hydration state of the plasmids contributed to the differences observed. Since the AFM can operate under a variety of conditions it may prove to be a very useful tool for studying changes in molecular conformation as a function of changes in chemical environment.

Several recent studies have shown that it is possible to image and manipulate biological and non-biological molecules with the AFM [3, 6–12, E. Henderson, unpublished data]. This report shows that it is possible to remove small regions of individual supercoiled plasmids by adjusting the force used during imaging. This is accomplished readily on hydrated supercoiled plasmids that are deposited on mica but much less so on desiccated samples. The price for this ability is reduced imaging resolution and sample stability. However, reduced sample stability may prove beneficial for recovery of small excised DNA fragments. Although the dissection shown here is crude by AFM standards, it is roughly five times more precise than microdissection techniques currently used to isolate specific chromosome fragments for subsequent cloning and sequence determination [4]. Moreover, a single

molecule, rather than highly amplified polytene chromatin or condensed metaphase chromosomes, is the subject of the dissection. Given a mechanism to localize sites of interest in euchromatin, the data presented here suggest that individual gene isolation and manipulation is possible by AFM. In principle, the resolution of the scission is limited to the resolution of the piezo crystal controlling tip movement and by the tip size and sharpness. Since the AFM can image atomic features it is feasible that atomic scale surgery can be accomplished by this instrument. In view of the resolution of the AFM and the precision of the dissection, the term 'nanodissection' is suggested for this procedure.

Current development in AFM suggest means by which excised portions of molecules may be more precisely manipulated and recovered. For example, positively charging of the scanning tip, possible in the electrochemical AFM [13], should enable one to retrieve the excised piece of DNA via electrostatic interactions between the DNA and the tip, and deliver it to a specific position or receptacle. In a combined visible light/fluorescence/atomic force microscope, one would then be able to dissect very fine regions of DNA from chromatin in an orderly fashion and rapidly determine the order of the sequences thus isolated by standard techniques. Recent work even hints at the possibility of directly sequencing the DNA by AFM [14]. This would be a significant contribution to genome sequencing projects.

ACKNOWLEDGEMENTS

The author would like to acknowledge Helen Hansma, Jan Hoh, James Vesenka, Philip Haydon and Drena Larson for criticisms, comments and discussions that helped improve this work. The author was supported by grants from the NSF (DIR-9004649), NIH (GM41023), and ACS (JFRA240).

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